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Insect feeding-induced differential expression of *Beta vulgaris* root genes and their regulation by defense-associated signals

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Abstract Root responses to insect pests are an area of plant defense research that lacks much information. We have identified more than 150 sugar beet root ESTs enriched for genes responding to sugar beet root maggot feeding from both moderately resistant, F1016, and susceptible, F1010, genotypes using suppressive subtractive hybridization. The largest number of identified F1016 genes grouped into the defense/stress response (28%) and secondary metabolism (10%) categories with a polyphenol oxidase gene, from F1016, identified most often from the subtractive libraries. The differential expression of the root ESTs was confirmed with RT-PCR. The ESTs were further characterized using macroarray-generated expression profiles from F1016 sugar beet roots following mechanical wounding and treatment of roots with the signaling molecules methyl jasmonate, salicylic acid and ethylene. Of the examined root ESTs, 20, 17 and 11% were regulated by methyl jasmonate, salicylic acid and ethylene, respectively, suggesting these signaling pathways are involved in sugar beet root defense responses to insects. Identification of these sugar beet root ESTs provides knowledge in the field of plant root defense and will lead to the development of novel control strategies for control of the sugar beet root maggot.

Keywords *Beta vulgaris* · Plant–insect interactions · Roots · Sugar beet root maggot · *Tetanops myopaeformis*

Abbreviations EST: Expressed sequence tagged · JA: Jasmonic acid · MJ: Methyl jasmonate · PR: Pathogenesis-related · SA: Salicylic acid · SBRM: Sugar beet root maggot · SSH: Suppressive subtractive hybridization

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Introduction

Plant defense studies have become a major part of the plant sciences with most of the focus on aerial parts of the plants and their interactions with microbes. Only more recently have insect pests become a larger part of molecular plant defense research (see reviews Baldwin et al. 2001; Kaloshian 2004; Kaloshian and Walling 2005; Stotz et al. 1999). Responses to herbivores include the production of plant volatiles capable of attracting parasitoids and signaling nearby plants (Arimura et al. 2005). Plant root studies are even less well-represented but have included sorghum expression profiles following chemical elicitor treatment (Salzman et al. 2005), the monitoring of terpenoid aldehyde accumulation in cotton exposed to wireworm root herbivory (Bezemer et al. 2004), the attraction of entomopathogenic nematodes to insect damaged maize roots (Rasmann et al. 2005), Douglas fir root–fungus interactions (Zamani et al. 2004), spinach–insect interactions (Schmelz et al. 1999) and defense responses of the grape–phylloxera interaction (Kellow et al. 2004) as well as root–nematode interactions (Bird 2004; Bird and Kaloshian 2003; Williamson and Gleason 2003).

Plant defenses are thought to be controlled in general by three major signaling molecules, jasmonates, salicylic acid and ethylene. These signaling pathways crosstalk with each other in order to respond appropriately to each pathogen (see review Feys and Parker 2000). Changes in gene expression underlying inducible responses to pathogens are known to be complex and multifaceted (Glazebrook 1999), and studies of responses to herbivory and mechanical wounding suggest a similar pattern of multiple independent, but networked defense response pathways (Hui et al. 2003; Korth and Dixon 1997; Puthoff et al. 2003; Reymond et al. 2000, 2004; Veena et al. 2003;

Voelckel and Baldwin 2004; Walling 2000). More specifically, changes in gene expression after herbivory do not always reflect that of jasmonate or wounding treatment (Reymond et al. 2000, 2004) and, in addition, herbivores with different feeding styles induce both unique and overlapping changes (De Vos et al. 2005). Knowledge of root responses at the molecular level will be useful in the development of alternative root insect control strategies for crop species.

An important insect pest of *Beta vulgaris* in the US and Canada is the sugar beet root maggot (SBRM), *Tetanops myopaeformis* von Röder. It inflicts yield losses ranging from 10 to 100% when the newly hatched larvae start feeding on lateral roots and the main taproot, which under severe infestations can be completely severed (Campbell et al. 1998; Cooke 1993; Dregseth et al. 2003). Damage incited by larval feeding consists of deformed root structure and secondary pathogen invasion both resulting in reduced yield and/or quality. No completely resistant germplasm has been identified and the currently available lines reduce the SBRM damage ratings by approximately 40% (Campbell et al. 2000). The most effective controls for this insect pest have been insecticide treatments (Mahrt and Blickenstaff 1979; Yun 1986).

We utilized the sugar beet root–SBRM system to study molecular root–insect interactions. This report presents the identification and characterization of more than 150 sugar beet root ESTs. These ESTs were identified using the suppressive subtractive hybridization (SSH) technique following SBRM infestation of sugar beet roots. Intra-genotype comparisons were conducted in both susceptible (F1010) and moderately resistant (F1016) sugar beet genotypes. Expression profiles of these ESTs were generated by analyzing macroarrays probed with labeled cDNAs from F1016 sugar beet roots wounded or roots treated with methyl jasmonate (MJ), salicylic acid (SA) or ethylene.

Materials and methods

Plant material, insects, and insect infestations

Beta vulgaris L. lines F1010 (Campbell 1990) and F1016 (Campbell et al. 2000) were obtained from Dr. Larry Campbell (USDA-ARS, Northern Crop Science Laboratory). The F1016 breeding line was developed from the cross between F1010 and another breeding line of unknown origin (Campbell et al. 2000). Seeds were grown 2–3 weeks in growth chambers under 16:8 (day:night) light regime at 24°C in soil.

Tetanops myopaeformis (SBRM) eggs of mated flies and second-instars collected from fields near St. Thomas, ND (Pembina County) were obtained from Dr. Mark Boetel (North Dakota State University). First-instar maggots were newly hatched from eggs incubated at 25°C for 1–2 days.

For insect infestations, 15 seedlings were washed to remove the soil and placed on 150 mm × 10 mm water/agar (0.8%) plates. Five first- or second-instar SBRM were placed on the root of each seedling and allowed to feed for 24 or 48 h. At the time of harvest, roots plus a small amount (<1 cm) of hypocotyl tissue were separated from the rest of the seedling, rinsed with water to remove SBRM larvae, and the tissues were frozen in liquid nitrogen and stored at −80°C until RNA isolation. The tissue consisted of a mixture of first- and second-instar infestations with 65 and 86% of the root tissues coming from seedlings infested with second-instar maggots for F1016 and F1010, respectively.

Chemical treatments and wounding

Sugar beet seeds (F1016) were planted as above. Soil was removed from the roots and seedlings were placed in plastic containers with 50 mM $NaPO_4$ (pH 7.0) supplemented with either 1 mM salicylic acid, 100 μM methyl jasmonate (Thurau et al. 2003) or 1 mM Ethephon (Mazarei et al. 2002), which slowly releases ethylene as a result of a chemical reaction. Roots for wounding treatment were crushed with forceps every centimeter. Control plants were treated identically except they were not wounded nor were elicitors added to the phosphate buffer. After 24 and 48 h, roots were harvested as above. Two independent biological experiments were conducted.

RNA isolation

Frozen root tissue was ground to a fine powder under liquid nitrogen. Total RNA was isolated as previously documented (Stiekema et al. 1988) with the following modifications. In brief, to 300 mg of frozen tissue 500 μl of extraction buffer (0.2 M NaOAc, pH 5.2; 1% SDS; 0.01 M EDTA; 0.5 mg/ml heparin; 0.02 M 2-mercaptoethanol) and 500 μl water-saturated re-distilled phenol was added followed by vigorous vortexing. The organic phase was re-extracted with 200 μl of extraction buffer, centrifuged as before and the two aqueous phases combined. The aqueous phase was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with chloroform:isoamyl alcohol (24:1). Total RNA was precipitated with 0.33 volumes of 10 M LiCl at −80°C for 1 h. Total RNA was resuspended in water and quantified spectrophotometrically. RNA quality was assessed using denaturing agarose/formaldehyde gel electrophoresis. PolyA^+ RNA was purified using DynaBeads (Dynal, www.invitrogen.com) according to manufacturer's instructions and quantified spectrophotometrically.

SSH and differential hybridization

Suppressive subtractive hybridization (SSH) was conducted using the PCR-Select cDNA Subtraction Kit (BD Biosciences, www.bdbiosciences.com) as described in manufacturer's instructions with 2 μg polyA^+ RNA. Three complete subtractions were conducted: (1) F1010 SBRM infested vs. uninfested, (2) F1016 SBRM infested vs. uninfested, and (3) F1010 vs. F1016 with both uninfested and SBRM infested tissues (see Table 1). The tissue used

Table 1 Sugar beet root tissues used for the suppressive subtractive hybridization (SSH)

Sugar beet variety	Treatment	Treatment (h)	Intra-genotype subtractions	No. of clones picked	Inter-genotype subtractions	No. of clones picked
F1010	Uninfested	24	Forward[a] and Reverse[b] subtraction	384	Forward[c] and Reverse[d] subtraction	288
		48				
	SBRM	24				
		48				
F1016	Uninfested	24	Forward[a] and Reverse[b] subtraction	384		
		48				
	SBRM	24				
		48				

[a]The forward subtraction consisted of cDNA from SBRM infested tissue as the tester and cDNA from uninfested tissue as driver

[b]The reverse subtraction consisted of cDNA from uninfested tissue as the tester and cDNA from SBRM infested tissue as driver

[c]The forward subtraction consisted of infested and uninfested cDNA from F1016 as the tester and infested and uninfested cDNA from F1010 as driver

[d]The reverse subtraction consisted of infested and uninfested cDNA from F1010 as the tester and infested and uninfested cDNA from F1016 as driver

for each subtraction was a pool of at least three biological replicate experiments. The resulting subtractive libraries were cloned in pCR2.1 TOPO (Invitrogen) and transformed into TOP10 cells (Invitrogen). Clones were plated on LB media supplemented with kanamycin (50 μg/ml; LB_{kan}), single colonies were picked into 96-well plates containing LB_{kan}, grown overnight, supplemented with an equal volume of 60% glycerol and frozen at −80°C.

Differential expression confirmation was conducted as directed using the PCR-Select Differential Screening Kit (BD Biosciences) using the same RNA as was used for the SSH procedure. In brief, 100 μl cultures in LB_{kan} were grown for 7.5 h at 37°C, 2 μl of culture was used as template for insert amplification. Amplification success was confirmed with gel electrophoresis. Two microliters of the PCR reaction was denatured, spotted onto nylon membrane using a 12-channel pipette and neutralized in 0.5 M Tris–HCl (pH 7.0). Membranes were dried, UV cross-linked and stored under vacuum until hybridization. Forward and Reverse subtracted probes were synthesized using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, www.roche-applied-science.com) as directed in the differential screening kit. Probes were quantified as directed in the manufacturer's instruction (DIG-High Prime DNA Labeling and Detection Starter Kit II; Roche) in order to ensure equal amounts of probe were used in all hybridizations. Pre-hybridization and hybridization were conducted at 42°C for 2 and 16 h, respectively in DIG Easy Hyb Granules (Roche) supplemented with Blocking Solution, as directed (PCR-Select Differential Screening Kit, BD Biosciences) and 0.0625 μg/ml sheared, denatured herring sperm DNA. Blots were washed 2–10 min in 2 × SSC/0.1% SDS at room temperature and 2–15 min in 0.35 × SSC/0.1% SDS at 65°C. Detection of DIG probes was carried out as directed using CSPD Ready-to-Use (DIG-High Prime DNA Labeling and Detection Starter Kit II; Roche) except blots were incubated with Blocking buffer (supplied in kit) for 1 h instead of 0.5 h. Images of the chemiluminescence were gathered using the AlphaImager 3400 (AlphaInnotech, San Leandro, CA). Clones visually identified as differentially regulated were picked into new 96-well plates, grown overnight in LB_{kan}, supplemented with glycerol as above and used as master plates for sequencing.

Sequencing and BLAST

Sequencing of differentially expressed clones was carried out at the DNA Synthesis and Sequencing Facility, Iowa State University, Ames, IA. Raw sequences were stripped of contaminating vector sequence and subjected to batch BLASTX (Altschul et al. 1997) against the GenBank non-redundant database. Batch BLASTN was also conducted against the TIGR *Beta vulgaris* gene index (BvGI) in order identify sugar beet ESTs. Individual clones were compared to each other, using local BLASTN, to identify a unique set of ESTs. A representative individual clone of each gene was picked into new 96-well plates, frozen as a glycerol stock and used as the macroarray master plate.

Inserts from the macroarray master plate set of clones were PCR amplified as above. Controls included on the macroarrays were dilutions of a Detection Control (DIG-High Prime DNA Labeling and Detection Starter Kit II; Roche) to monitor for film exposure and blank spots to control for background hybridization. In brief, 5 μl of the PCR reaction, 190 μl H_2O, 210 μl 0.4N NaOH were mixed at room temperature. Using a 96-well dot blotter (Bio-Dot, Bio-Rad, www.biorad.com) 100 μl was spotted onto each of four blots. After liquid was pulled through the nylon membrane 200 μl of 0.4 M NaOH, and 200 μl 2 × SSC were sequentially pulled through each well. The membranes were then transferred to filter paper presoaked with 0.5 M Tris–HCl (pH 7.0) for 4 min and air dried. DNA was UV cross-linked to the membrane with 4 min of exposure to UV light from the gel box used for imaging ethidium bromide stained gels. Membranes were stored under vacuum at room temperature until hybridization. Two experiments were conducted and clones were spotted once

in the first experiment or twice in different areas of the nylon membrane in the second experiment.

Macroarray hybridization, detection and imaging

RNA from treated and control samples was purified as above. First strand cDNA was synthesized with Superscript II (Invitrogen) as directed by the manufacturer except the oligo d(T) primer contained a T7 RNA polymerase binding site (5′-TAATACGACTCACTATAGGGATTTTTTTTTT TTTTTTTT-3′). Second strand cDNA synthesis was conducted with the addition of 91 μl water; 30 μl 5 × second strand buffer (Invitrogen); 3 μl 10 mM dNTPs; 1 μl *E. coli* DNA ligase; 4 μl DNA polymerase I and 1 μl RNase H to each reaction. After 2 h at 16°C, 2 μl of T4 DNA polymerase was added followed by an additional incubation at 16°C for 5 min. Following phenol:chloroform extraction and EtOH precipitation with 10 μg glycogen, cDNA was subjected to amplification using the Megascript Kit (Ambion, www.ambion.com) as directed by the manufacturer. Copy RNA was LiCl precipitated according to the Megascript instructions. The resulting RNA was converted into dscDNA as above using random primers (250 ng) (IDT, www.idtdna.com) instead of oligo d(T). This cDNA was phenol/chloroform extracted and EtOH precipitated with glycogen and resuspended in water. Probes were synthesized using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche), purified using Wizard PCR Preps DNA Purification System (Promega, www.promega.com) and were quantified as directed in the manufacturer's instruction in order to ensure equal amounts of probe were used. Pre-hybridization and hybridization were conducted at 42°C for 3 and 20 h, respectively, in DIG Easy Hyb granules. Blots were washed and detected as above and exposed to film for varying amounts of time (1–10 min) to obtain an optimal exposure resulting in the visualization of most spots while minimizing the over-exposure of spots. The resulting films were scanned and quantified using ImageQuant TL software (Amersham Biosciences, www1.amershambiosciences.com). Comparisons between blots were conducted using the Standardization and Normalization of MicroArray Data (SNOMAD) website: http://pevsnerlab.kennedykrieger.org/snomadinput.html using function nos. 2, 2optional, 3, 4, 5 and 6. The raw data gathered from these experiments is in compliance with the MIAMI standards and is available in Table S2.

For each clone, three separate fold-change values were calculated (one from the first hybridization/biological replicate and two from the second hybridization/second biological replicate). These three separate fold change values were then used to calculate an average fold change value as follows. All three of the values for a particular clone had to meet one of the following criteria: (i) all three positive, (ii) all three negative, or (iii) all three unchanged ($-1.5 < x < 1.5$). If one of these criteria were met, an average of the fold change values was calculated. This final average fold change, which is reported in Tables 3 and 4, was considered biologically relevant if it was either >1.5 or <-1.5. If the three fold change values did not meet one of the above criteria, no final fold change value was calculated and the gene was considered as not responding to the plant treatment.

RT-PCR

Total RNA from the same tissue used for SSH, was DNase treated (DNA-free, Ambion). First strand cDNA was synthesized using SuperScript II (Invitrogen) as directed by the manufacturer. Negative controls consisting of cDNA synthesis reactions without reverse transcriptase were also conducted to rule out genomic DNA as a source of template for PCR reactions. The remaining RNA was hydrolyzed with the addition of NaOH to a final concentration of 0.024 M, incubation at 70°C for 10 min and neutralization with the addition of an equal Normal volume of HCl. First strand cDNA was precipitated with 0.1 volume 3 M sodium acetate, 10 μg glycogen and 2.5 volumes of 100% ice-cold ethanol. Following centrifugation to pellet cDNA and two 70% EtOH washes, cDNA was dried and resuspended in water to 10 ng/μl. In order to ensure equal amounts of cDNA template were placed in each PCR reaction, cDNA was quantified spectrophotometrically and confirmed visually with ethidium bromide (EtBr) staining. PCR reactions were set up as follows: 10 ng template cDNA; 1 × buffer; 0.25 mM each dNTP; 0.25 μM each primer (specific for individual genes); 0.625 U of HS ExTaq (Takara, www.takara-bio.com); water to 30 μl. Aliquots of PCR reactions were removed from tubes after 15, 20, 25, 30 and 35 cycles. The basic thermocycling parameters were as follows: an initial incubation at 94°C, 30 s; followed by 94°C, 10 s; annealing temperature specific for each gene (53–58°C), 40 s; 72°C, 1 min. PCR products were separated on 1.5% agarose gels, stained with EtBr and images were captured with an AlphaImager as above and quantified using ImageQuant TL (Amersham Biosciences).

Results

SSH

We used the SSH methodology to enrich for genes that are regulated by SBRM feeding and may be involved in the defense responses of the root to gain a better understanding of root–insect interactions and resistance mechanisms. Three complete subtractive procedures were carried out. As shown in Table 1, both SBRM susceptible (F1010) and moderately resistant (F1016) sugar beet varieties were used (Campbell 1990; Campbell et al. 2000). Forward subtractions (infested cDNA as tester; uninfested cDNA as driver) which enrich for up-regulated genes and reverse subtractions (infested cDNA as driver; uninfested cDNA as tester) which enrich for down-regulated genes were conducted within each genotype and infestations were with first- and second-instar SBRM. Second-instars were

used in combination with first-instar larvae because of their larger physical size which manifested into more inflicted damage. A pooled sample from 24 and 48 h time points was compared to a pooled sample from uninfested tissue. These subtractions allow the cloning of F1010 and F1016 genes that may be regulated by SBRM feeding. The number of clones picked from each subtraction is listed in Table 1. Approximately, 800 clones were picked from the two intra-genotype libraries (F1010 and F1016) and subjected to differential hybridization.

A third comparison was also made between the two genotypes (Table 1). For this inter-genotype subtraction, all four samples (uninfested and SBRM infested) from F1010 were pooled and compared in both forward and reverse directions to all tissue samples pooled from similarly treated F1016. These subtractions produced fewer clones, as expected, but similar comparisons have been shown to identify additional genes not detected by intra-genotype comparisons (Puthoff et al. 2003).

Differential screening

Differential expression of the cloned SSH libraries was confirmed using a labeling and detection method that gave bright signals with relatively low background (Zhang et al. 2002). As expected, individual clones hybridized only with the expected probe. For example, clones picked from the forward subtraction of F1016 hybridized only to the forward subtracted probe (data not shown). Approximately, 60% of the screened clones showed differential hybridization between the forward and reverse probes across all three subtractive procedures.

Clones confirmed to be differentially expressed were sequenced to determine insert size and putative function based on sequence similarity. As expected, most clones contained relatively short inserts with an average insert size of 537 bp over all three subtractions. The intra-genotype subtractions using the moderately resistant F1016 genotype identified 121 unique genes (71 forward subtraction; 50 reverse subtraction) (Table 2) while the intra-genotype subtractions of SBRM susceptible F1010 identified 42 unique SBRM regulated genes (14 forward subtraction; 28 reverse subtraction) (Table S1). Only five genes were identified from the inter-genotype subtraction when F1016 cDNA was used as the tester (forward subtraction, Table S1). In contrast, 41 genes were identified from the inter-genotype reverse subtraction (Table S1).

Clones were functionally annotated based on batch BLASTX results and grouped accordingly. Figure 1 shows the breakdown of F1016 and F1010 clones from the intra-genotype subtractions into their respective categories. While many functional categories are represented, the largest number, excluding clones with no known function, falls into the defense-related class where 28 (35 of 121) and 21% (9 of 43) of F1016 and F1010 clones, respectively, were found. The list of genes identified in the F1016 subtraction is listed in Table 2 along with putative function, number of times identified in the SSH library and EST similarities. Complete lists of genes identified from the F1016, F1010 and inter-genotype subtractions along with GenBank identification numbers, insert length, putative function, e-values and best sugar beet EST hits are included as supplemental data in Table S1.

RT-PCR confirmation of SSH procedure

An additional confirmation of the differential expression of 17 genes identified using the SSH procedure (Table 3) was carried out using RT-PCR. These genes were chosen to represent both forward and reverse subtractions from all three comparisons, but were otherwise chosen at random. First strand cDNA was synthesized and used as template in each PCR reaction.

Ten genes representing the F1016 subtraction were assessed with RT-PCR (DV501541, DV501635, DV501606, DV501607, DV501611, DV501521, DV501691, DV501692, DV501590, DV501610). Eight of the clones showed total agreement between SSH and RT-PCR (Fig. 2a). Two of the clones (DV501606, DV501607) that were identified in the reverse subtraction (i.e. down-regulated in infested tissue) showed either no change in expression or a slight up-regulation, respectively, using RT-PCR analyses (Fig. 2a).

Four genes representing the F1010 subtraction were analyzed with RT-PCR and three, DV501924, DV501888 and DV501887, were up- and down-regulated as predicted by SSH (Fig. 2b). Of these three genes, DV501924 was also shown by RT-PCR to be up-regulated in F1016 after SBRM infestation but was not found as one of the F1016 unique clones identified with SSH. Interestingly, DV501924 shares homology with pathogenesis-related (PR) protein 10 and is expressed at a higher level in F1016 when compared to F1010, suggesting it plays a role in resistance. The F1010 clone DV501971 that was identified in the reverse subtraction showed no change in transcript abundance by RT-PCR analysis.

For the inter-genotype subtractions, clone DV501749 was identified in the forward subtraction and shown by RT-PCR to be up-regulated (Fig. 2c). A random clone, DV501776**,** picked from the forward subtraction but not shown to be regulated after differential hybridization was up-regulated 1.4-fold between the two genotypes using RT-PCR. The final gene, DV501807 was identified as up-regulated in the F1016 by F1010 subtraction, but was shown by RT-PCR to be minimally down-regulated (-1.2-fold).

Negative control reactions lacking reverse transcriptase were conducted to ensure the absence of contaminating genomic DNA. PCR from these negative control reactions did not produce any bands (data not shown). In addition, although the RT-PCR data were mostly unreplicated, they largely confirmed the utility of SSH for the identification of genes differentially regulated by SBRM feeding. Quantitative data on gene expression changes, however, were generated using macroarrays.

Table 2 Sugar beet ESTs from the moderately resistant to SBRM genotype F1016 identified by SSH and ordered based on the number of times identified

GenBank ID	No. of times found	Homology	e-value of BLASTX hit	Best sugar beet EST hit (TIGR)	e-value of EST hit
Forward subtraction					
DV501668	7	Polyphenol oxidase	e-40	TC1454	e-165
DV501577	7	Hypothetical protein – tomato	e-48	BQ488328	e-44
DV501585	7	Aspartate aminotransferase	e-18	TC1617	e-165
DV501660	4	Osmotin 81 (*Solanum*)	e-36	TC1084	0.0
DV501651	4	14-3-3 d-2-AS protein	e-21	TC3265	0.0
DV501620	4	Notch-1 (*Mus musculus*)	e0.041	BQ591913	0.0
DV501542	3	Auxin-induced beta-glucosidase	e-27	TC2835	e-6
DV501565	3	Chitinase, class V	e-08		
DV501547	3	No hits		TC1685	e-125
DV501628	2	Glutathione *S*-transferase	e-41	BQ592393	e-137
DV501633	2	Polyphenol oxidase	e-43	TC508	e-08
DV501644	2	Oxalate oxidase-like germin 165	e-34	NP1332951	e-30
DV501683	2	Plastid-targeted protein 3	e-13		
DV501520	2	Stress-induced cysteine proteinase	e-50		
DV501519	2	psaH (*Spinacia*)	e-50	BQ586570	0.0
DV501524	2	Branched-chain alpha keto-acid dehydrogenase	e-56	BQ585029	0.0
DV501659	2	Ntdin (*Nicotiana*)	e-42	BQ585573	e-171
DV501611	2	No hits		TC2626	e-123
DV501571	2	No hits		TC1763	e-115
DV501596	2	No hits			
DV501622	1	Translation initiation factor (eIF-1A)	e-49	TC1312	0.0
DV501536	1	Elongation factor 1A SMV resistance-related	e-09	TC5	e-67
DV501558	1	Putative 60S ribosomal protein L9	e-40	TC73	e-159
DV501699	1	Glutathione *S*-transferase	e-16	BQ591317	e-122
DV501540	1	Chitinase	e-116	TC256	0.0
DV501610	1	Jasmonate-induced protein homolog	e-08	BQ595274	e-114
DV501636	1	Polyphenol oxidase	e-56	TC2792	0.0
DV501637	1	Putative xyloglucanase inhibitor	e-34	BQ586566	e-165
DV501657	1	Beta-glucosidase	e-109	TC2747	e-131
DV501666	1	Xyloglucan endotransglucosylase	e-87	BQ586000	0.0
DV501669	1	Class I chitinase	e-62	BQ588674	0.0
DV501673	1	Osmotin-like protein	e-17	TC1084	e-127
DV501680	1	Polyphenol oxidase	e-32	TC1454	e-48
DV501681	1	Polyphenol oxidase	e-23		
DV501688	1	Kunitz-type trypsin inhibitor	e-14	BQ585721	e-18
DV501518	1	Auxin-induced beta-glucosidase	e-25		
DV501528	1	Drought-induced protein SDi-6	e-12	TC1132	0.0
DV501572	1	SAR1/GTP-binding secretory factor	e-66	TC1219	e-46
DV501603	1	Putative 32.7 kDa jasmonate-induced protein	e-07	TC2716	e-179
DV501653	1	Glutathione *S*-transferase 2	e-16	TC3597	e-67
DV501630	1	Annexin	e-18	TC2850	e-125
DV501621	1	Hypothetical protein – tomato	e-51	BQ588546	e-10
DV501563	1	Unnamed protein product	e-30		
DV501649	1	Barley B recombinant	e0.12	TC17	0.0
DV501667	1	Rubisco activase	e-67	TC2796	0.0

Table 2 Continued

GenBank ID	No. of times found	Homology	e-value of BLASTX hit	Best sugar beet EST hit (TIGR)	e-value of EST hit
DV501689	1	Geranylgeranyl hydrogenase	e-89	TC1764	e-73
DV501609	1	12-Oxophytodienoate reductase 3	e-35		
DV501618	1	Asparagine synthetase	e-18		
DV501635	1	Cytosolic 6-phosphogluconate	e-109	TC1740	0.0
DV501642	1	Raffinose synthase family protein	e-34	BQ591930	e-13
DV501555	1	UDP-galactose 4-epimerase	e-53		
DV501601	1	Flavonol 4′-sulfotransferase	e-22		
DV501674	1	Hydrolase, alpha/beta fold family protein	e-05		
DV501516	1	Nucleic acid binding protein-like	e-29		
DV501526	1	Putative receptor-like protein kinase 4 RLK4	e-75	TC3541	0.0
DV501535	1	Transcription factor jumonji (jmjC)	e-48		
DV501541	1	BTB and TAZ domain protein 2	e-32		
DV501614	1	Dioscorin A	e-27	TC3225	0.0
DV501617	1	J2P (*Daucus carota*)	e-15	TC2708	0.0
DV501533	1	Specific tissue protein 2 (*Astragalus*)	e-07	CV301908	0.0
DV501574	1	Glycosyl hydrolase family 3 protein	e-08	TC2696	e-49
DV501579	1	Major latex-like protein homolog	e-12	BQ592126	0.0
DV501580	1	Major latex-like protein homolog	e-33	TC169	0.0
DV501581	1	Kelch repeat-containing F-box family protein	e-38	BQ583945	e-17
DV501593	1	GA (*Pisum sativum*)	e-04		
DV501677	1	Peroxisomal ascorbate peroxidase	e-52	TC203	0.0
DV501691	1	Ubiquitin-conjugating enzyme E2	e-57	BF011151	e-53
DV501534	1	No hits			
DV501548	1	No hits			
DV501549	1	No hits			
DV501570	1	No hits			
Reverse subtraction					
DV501693	5	LHCII type III chlorophyll a/b binding protein	e-108	TC462	0.0
DV501521	4	Glutathione *S*-transferase	e-61	TC589	0.0
DV501605	3	Jasmonate-induced protein homolog	e-23	TC3604	0.0
DV501655	3	Ribulose-bisphosphate carboxylase	e-33	TC1343	0.0
DV501553	3	Calcium-binding EF hand-like protein	e-82	BQ584282	0.0
DV501559	2	Putative 40S ribosomal protein S2	e-49		
DV501608	2	Ribosomal protein L2	e-92	TC94	0.0
DV501670	2	Cysteine protease	e-63		
DV501700	2	Chlorophyll a/b binding protein	e-51	TC1363	e-139
DV501624	2	Chlorophyll a/b binding protein	e-148	TC2698	0.0
DV501607	2	5′-Aminoimidazole ribonucleotide synthetase	e-08	BQ587122	0.0
DV501692	2	Uracil phosphoribosyltransferase	e-53		
DV501664	2	P-Protein precursor	e-98	TC2951	e-175
DV501531	2	KH domain-containing protein-like	e-43	TC3217	e-13

Table 2 Continued

GenBank ID	No. of times found	Homology	e-value of BLASTX hit	Best sugar beet EST hit (TIGR)	e-value of EST hit
DV501522	1	60S ribosomal protein L10	e-53	TC2736	e-174
DV501523	1	Eukaryotic elongation factor 1A	e-88	TC5	0.0
DV501529	1	Eukaryotic translation initiation factor 2B family	e-59	BQ587194	e-160
DV501537	1	Alpha-tubulin	e-64	TC6	e-44
DV501546	1	40S ribosomal protein S19-like	e-35	TC79	e-169
DV501568	1	Cytosolic ascorbate peroxidase 1	e-20	TC1366	0.0
DV501576	1	Histone H2B	e-07		
DV501591	1	Phosphoenolpyruvate carboxylase	e-73	TC1867	0.0
DV501592	1	Acetoacetyl-coenzyme A thiolase	e-59	TC565	e-47
DV501530	1	60S ribosomal protein L10	e-34	TC2736	e-80
DV501632	1	Peroxidase	e-60		
DV501639	1	Plastidic aldolase NPALDP1	e-99	TC413	e-171
DV501590	1	Subtilisin-like serine protease	e-60	BQ589176	e-73
DV501606	1	Polyphenol oxidase	e-20		
DV501646	1	Unknown protein (*Arabidopsis*)	e-70	BQ585818	0.0
DV501539	1	Unknown protein	e-77	BQ487685	0.0
DV501552	1	Expressed protein	e-09	BQ593046	0.0
DV501631	1	Phosphoribulokinase	e-119	TC532	0.0
DV501702	1	Glyceraldehyde-3-phosphate dehydrogenase	e-90	TC1407	0.0
DV501679	1	Chlorophyll a/b binding protein	e-68	TC1379	0.0
DV501616	1	Ribisco small subunit	e-47	TC1344	e-154
DV501623	1	Ribulose bisphosphate carboxylase small subunit	e-14	TC1343	e-14
DV501640	1	Chlorophyll a/b binding protein type I	e-36	TC1430	e-125
DV501654	1	Chlorophyll a/b binding protein type I	e-43	TC2700	0.0
DV501551	1	PHO1 protein	e-64		
DV501662	1	Alanine aminotransferase	e-54	TC103	0.0
DV501597	1	Taxadiene 5-alpha hydroxylase	e-46	TC3574	e-18
DV501647	1	Protein phosphatase 2A regulatory subunit B	e-25	TC1568	0.0
DV501663	1	Ferredoxin-$NADP^+$ reductase	e-57	TC150	e-154
DV501567	1	WRKY transcription factor 65	e-100	BQ585499	e-06
DV501569	1	Putative nucleic acid binding protein	e-47	TC1719	0.0
DV501694	1	Cytochrome P450 monooxygenase	e-16	TC33	e-161
DV501561	1	UPF1 (*Arabidopsis*)	e-09		
DV501575	1	H(+)-transporting ATPase	e-103	TC832	0.0
DV501545	1	*S*-Adenosylmethionine sythetase 2	e-100	TC2715	0.0
DV501687	1	Unknown	e-48	BQ587027	e-115

Expression profiling using macroarrays

Macroarrays of the genes identified with SSH were used to elucidate gene expression changes in the moderately resistant F1016 roots following four plant treatments that included mechanical wounding and treatment with exogenous MJ, SA or ethylene. Each treatment was replicated with a second biological experiment and the results were combined. These experiments offer additional clues as to their biological function and role in root defense responses, specifically to SBRM. The clones included on the macroarray (163 total) represent genes identified from both F1010 and F1016 SSH libraries, were chosen based on putative function determined by sequence similarity and clone length and are listed in Table S2. The number of uniquely and co-regulated genes is shown in Fig. 3a and b. Supplemental data of intensity values and fold changes for all macroarrays are given in Table S2. To assess the imaging

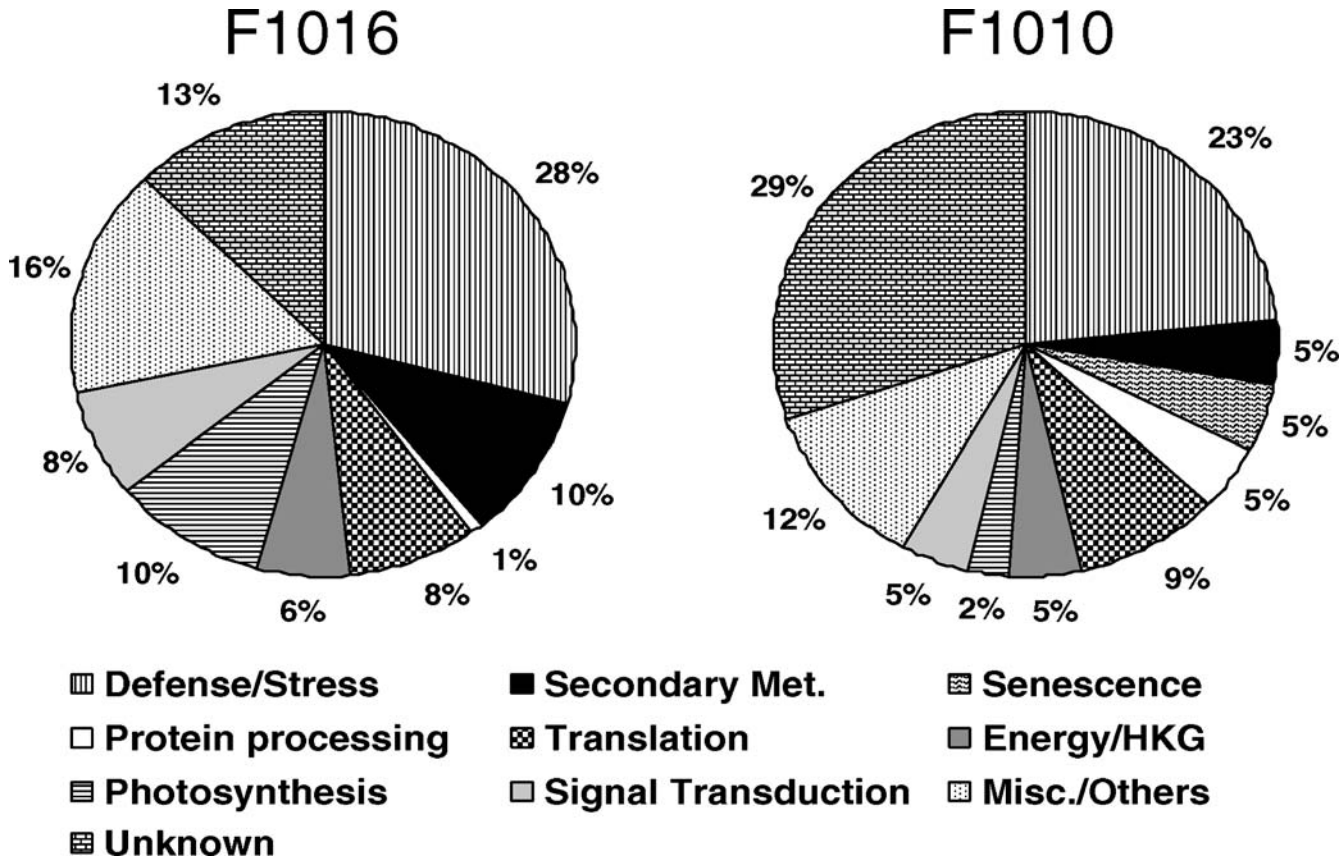

Fig. 1 Functional groups of sugar beet genes identified by SSH as responding to SBRM infestation. Genes identified with SSH from moderately resistant F1016 and susceptible F1010 genotypes were annotated using BLASTX and grouped according to the categories listed at the bottom. The percentage of ESTs falling into each category is shown. The total number of genes identified was 121 for F1016 and 42 for F1010

and quantification of the macroarrays, ten-fold dilutions of a detection control (provided in the labeling kit, see Materials and methods) were spotted in addition to the unique genes. A strong correlation was found between the arbitrary spotted quantity of the control and the quantified intensity ($R^2 = 0.96$, data not shown) and shows that our quantification resulted in accurate interpretations of the hybridized intensity.

Wounding of sugar beet roots regulated the expression of 36 out of 163 genes (14 up-regulated, 22 down-regulated) (Fig. 3a). Six genes were co-regulated by both wounding and MJ treatments (Table 4). Interestingly, each of the six co-regulated genes exhibited a similar pattern of regulation, induced after wounding and MJ treatment or down-regulated following both treatments.

MJ treatment of F1016 sugar beet roots altered the expression of 33 of the 163 genes on the macroarray (16 up-regulated, 17 down-regulated) (Fig. 3b). Similarly, both SA and ethylene (ethephon) treatments resulted in gene expression changes. While SA regulated 28 genes (12 up-regulated, 16 down-regulated), ethylene treatment only resulted in 18 altered transcript levels (12 up-regulated, 6 down-regulated) (Fig. 3b). Interestingly, while MJ and SA treatments showed approximately equal numbers of up- and down-regulated changes, treatment with ethylene resulted in a biased number of changes; heavily weighted toward up-regulation. The three signaling molecules were responsible for numerous unique gene expression changes, however, only four genes were co-regulated by all three treatments, i.e. MJ, SA, ethylene (Fig. 3b). Table 5 lists the putative functions of the four genes co-regulated by all three elicitors and by any two of the three signaling molecules. The four genes co-regulated by all three elicitors showed both positive and negative coordination. For example, clone DV501855 showed up-regulation of 2.4-, 2.4-, and 1.6-fold following MJ, SA and ethylene treatments, respectively (Table 5, first row). In contrast, clone DV501953 showed 1.8-fold up-regulation following MJ treatment but more than 6- and 2.8-fold down-regulation following SA and ethylene treatments, respectively (Table 5, second row).

Discussion

While the study of plant responses to insect pests has garnered greater interest as of late, the study of root feeding insects is far behind the extensive studies of foliar interactions (Hunter 2001). Damage from SBRM is a serious problem and their control relies primarily on chemical pesticides. The identification of genes following SBRM larval

Table 3 Functional annotation of the sugar beet root ESTs in Fig. 2

GenBank ID	Homology
ESTs from the F1016 intra-genotype subtraction	
DV501541	BTB and TAZ domain protein 2
DV501635	Cytosolic 6-phosphogluconate
DV501606	Polyphenol oxidase
DV501607	5′-Aminoimidazole ribonucleotide synthetase
DV501611	No hits
DV501521	Glutathione *S*-transferase
DV501691	Ubiquitin-conjugating enzyme E2
DV501692	Uracil phosphoribosyltransferase
DV501590	Subtilisin-like serine protease
DV501610	Jasmonate-induced protein homolog
ESTs from the F1010 intra-genotype subtraction	
DV501924	Pathogenesis-related protein 10
DV501888	Hypersensitive-induced response protein
DV501887	Elicitor-inducible protein EIG-J7
DV501971	Senescence-associated protein
ESTs from the inter-genotype subtraction	
DV501749	No hits
DV501776	Ribosomal protein L30
DV501807	*O*-Methyltransferase

feeding on moderately resistant varieties allows for the formulation of testable hypotheses to gain knowledge on root defense responses and the mechanism of resistance. This valuable information will lead to development of alternate control measures.

Sugar beet root ESTs identified following SBRM feeding

We identified more than 150 genes with SSH following SBRM feeding on both SBRM susceptible and moderately resistant sugar beet lines. While no quantitative data can be obtained from this procedure, the ease of the SSH technique and rapid completion provides a list of potentially regulated genes which can then be more extensively investigated to identify those responsible for resistance (Ramalingam et al. 2003). The SSH method has been used in many systems for identifying genes with altered expression levels (Gepstein et al. 2003; Guilleroux and Osbourn 2004; Louie et al. 2003; Veena et al. 2003; Wang et al. 2005). While no method of differential gene expression can identify all regulated genes, SSH is a reasonable choice given the lack of knowledge available for the sugar beet genome that does not benefit from large scale sequencing (e.g. *Arabidopsis*, rice, maize) and has major genetic differences within and between varieties (De Riek et al. 2001).

The SSH libraries we generated are enriched for genes involved in the initial responses of sugar beet roots to insect herbivory and is supported by our RT-PCR data. Our experimental system which utilizes tissues from a feeding bioassay (Smigocki et al. 2005, unpublished data) is capable of screening for SBRM resistance and thus reflects field-like conditions. The intra-genotype comparisons provide a starting point for further investigation while the inter-genotype comparison, i.e. susceptible vs. resistant, adds an additional level of data useful for the identification of genes that are reciprocally regulated (Puthoff et al. 2003). While not definitive, sequence similarity/identity at the amino acid level can aid in the identification of protein function and give a starting point for determination of its role in plant cells. With this in mind, several interesting points can be made from the putative functions of the genes identified by SSH after SBRM feeding. First, many of the genes identified with SSH following SBRM feeding have been found to be regulated by other pathogens including insect pests. These include a polyphenol oxidase gene, DV501680 (Voelckel and Baldwin 2004), a beta-glucosidase gene, DV501657 (van de Ven et al. 2000), a glutathione *S*-transferase gene, DV501628 (Reymond et al. 2000), a subtilison-like serine protease gene, DV501590 (Tornero et al. 1996) and osmotin-like protein genes (Zhu et al. 1995). This shows that our data is in line with what

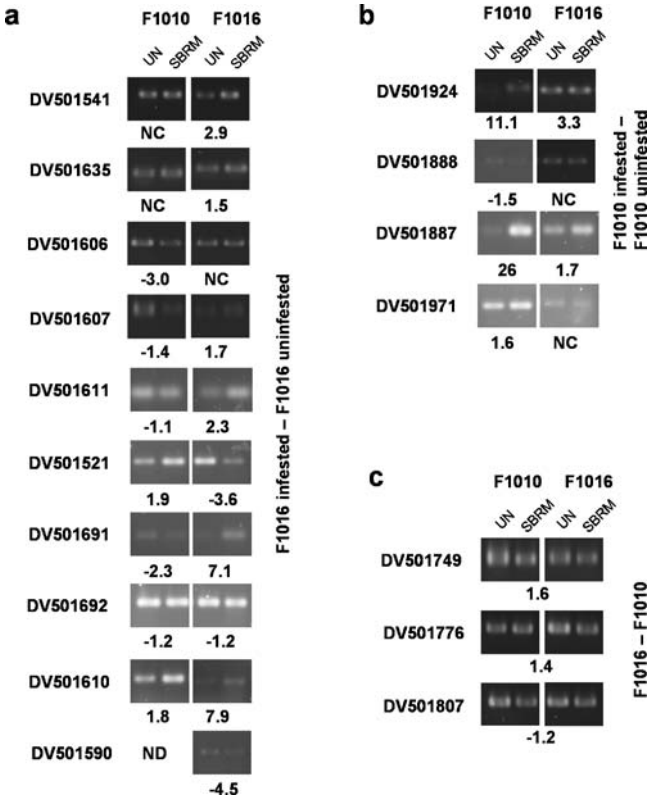

Fig. 2 RT-PCR expression analysis of genes identified with SSH. Following cDNA synthesis, gene specific primers were used to amplify transcripts identified from the **a** intra-genotype subtraction using F1016, **b** intra-genotype subtraction of F1010 and **c** the inter-genotype subtraction comparing F1010 and F1016. Fold changes between uninfested and infested samples are listed below each image. Fold changes in **c** were calculated by first combining the intensities of the two samples on each image. To ensure the PCR reactions were monitored within the linear phase of amplification, 25 PCR cycles, determined empirically, were used to generate these images except for DV501541 and DV501807, 30 cycles and DV501749, 20 cycles

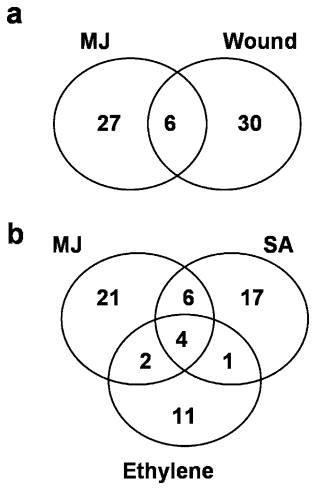

Fig. 3 Defense molecule treatment of sugar beet roots regulates specific and over-lapping gene sets. The number of uniquely and co-regulated genes identified using macroarray hybridization for **a** MJ and mechanical wounding and **b** MJ, SA and ethylene plant treatments. Two biological experiments with multiple spots for each clone were used to determine the set of regulated genes

other researchers have found and may suggest regulatory networks involved in signaling after SBRM infestation. Second, the regulation of PR protein genes was found and included a chitinase gene, oxalate oxidase-like genes, peroxidase genes, osmotin-like genes and a PR10-like gene. While PR protein genes are typically induced after microbe infections they have also been found to be regulated by insect feeding (Hui et al. 2003; Voelckel and Baldwin 2004). Third, we expected up-regulation of wound response genes such as proteinase inhibitors, lipoxygenases, and phenylalanine ammonia lyase. We identified, however, only one protease inhibitor (DV501688), with similarity to serine proteinase inhibitors. Interestingly, serine protease activity has been shown to be one of the major activities in SBRM larval guts (Wilhite et al. 2000). The identification of a serine protease inhibitor in only the moderately resistant F1016 genotype suggests it plays a role in resistance and warrants further investigation.

Similarly, we identified multiple polyphenol oxidase genes only in the resistant F1016 genotype subtractions. These enzymes are known to reduce the nutritive value of plant tissue for insects and are involved in secondary metabolite production (Felton et al. 1989; Sudha and Ravishankar 2002). In general, a greater number of genes involved in secondary metabolite synthesis was identified in the F1016 genotype with the SSH enrichment method (Fig. 1). These genes included one with similarity to taxadiene 5-alpha hydroxylase which is involved in the production of the secondary metabolite Taxol and is one of the initial steps in the production of diterpenoids (Wheeler et al. 2001), which suggests that diterpenoids are involved in sugar beet–SBRM interactions. Secondary metabolites may be associated with SBRM resistance since our preliminary evidence suggests that the resistance of the F1016 genotype is associated with the "attractiveness" of sugar beet roots to SBRM larvae (Puthoff and Smigocki unpublished results).

Table 4 Sugar beet root ESTs co-regulated by mechanical wounding and exogenous MJ treatment

GenBank ID	Homology	Fold change[a]	
		MJ	Wound
DV501862	ABI3-interacting protein 3; CnAIP3	1.6	30.9
DV501785	AER (*Nicotiana tabacum*)	2.0	22.3
DV501855	NIP3 (*Medicago*)	2.4	12.8
DV501692	Uracil phosphoribosyltransferase	9.0	8.0
DV501891	High mobility group protein 2 HMG2	− 2.7	− 1.9
DV501725	Salt tolerance protein 4 (*Beta vulgaris*)	− 1.5	− 3.7

[a]Fold changes listed were calculated using SNOMAD as detailed in Materials and methods

Comparison of genes identified in F1010 to those identified in F1016 did not reveal exact gene sequence matches. Common putative functions, however, were identified (Table S1), for example, a cysteine protease (best BLASTX hit for both) was identified in both F1010 and F1016 reverse subtractions. Similar results were obtained for an oxalate-oxidase gene that was identified in both intra-genotype forward subtractions. A closer examination of the respective genes would shed light on whether or not they are the same gene or members of a family that respond differently to environmental stimuli (Velazhahan et al. 1998; Wang and Constabel 2004).

Defense signal regulation suggests signaling pathways involved in root defense response

Given wounding, MJ, SA and ethylene have been well documented as signals and intricate parts of defense responses in plants, we evaluated their effects on the expression of the root genes identified with SSH. Our results reflect those of others including the induction of a PR-10 gene in sorghum roots after ethylene treatment and the down-regulation of a calcium-binding EF hand-like protein after jasmonate treatment (Salzman et al. 2005). Wounding and MJ treatments resulted in both unique and co-regulated gene expression changes with the two treatments co-regulating six genes. These findings are similar to those reported in previous studies (Constabel and Ryan 1998; Korth and Dixon 1997; León et al. 2001) which show both overlapping and specific gene regulation. It was interesting, however, to find that the pattern of regulation after wounding or MJ treatment was shared for each of the co-regulated clones. Each clone was either up-regulated by both wounding and MJ treatment or down-regulated by both treatments. It will be intriguing to more closely examine these and a more complete set of wound-regulated genes in order to confirm or refute these patterns of expression.

Table 5 Expression level changes in F1016 sugar beet root genes co-regulated by MJ, SA and ethylene treatment

GenBank ID	Homology	Fold change[a,b]		
		MJ	SA	Ethylene
DV501855	NIP3 (*Medicago*)	2.4	2.4	1.6
DV501694	Cytochrome P450 monooxygenase	1.8	− 6.1	− 2.8
DV501887	Elicitor-inducible protein EIG-J7	− 1.7	− 3.8	− 2.5
DV501953	No hits	− 2.2	− 3.7	− 1.7
DV501862	ABI3-interacting protein 3; CnAIP3	1.6		1.5
DV501553	Calcium-binding EF hand-like protein	− 2.2	− 1.0	2.7
DV501974	23 kDa OEC protein		− 3.2	− 1.6
DV501863	Zinc finger (C3HC4-type RING finger)	2.3	2.5	
DV501785	AER (*Nicotiana tabacum*)	2.0	1.6	1.4
DV501763	Ozone-responsive stress-related protein-like	1.8	1.7	
DV501928	Catalase	− 1.7	− 1.8	− 1.4
DV501579	Major latex-like protein homolog	− 1.8	− 2.1	− 1.1
DV501637	Putative xyloglucanase inhibitor	− 1.9	2.1	1.1

[a]Fold changes listed were calculated using SNOMAD as detailed in Materials and methods. Only co-regulated gene expression changes greater than or equal to 1.5-fold or less than or equal to − 1.5-fold are highlighted

[b]Gene expression changes not listed indicate that the average fold change, as calculated in Materials and methods was not determined because the gene had variable expression between the three replicates

Comparisons were also made between the three major signaling molecules in plant defense (MJ, SA, ethylene). While all three treatments resulted in gene expression changes, exogenous MJ treatment regulated the greatest number of the monitored genes. Given these genes were originally isolated and are enriched for SBRM responsiveness suggests that MJ signaling is involved in regulating sugar beet's response to this insect pest. This is not surprising given MJ's involvement with wound signaling and SBRM's rasping feeding style. SA treatment of sugar beet roots regulated a similar number of genes which may reflect the fact that MJ and SA signaling can be antagonistic (Doares et al. 1995; Peña-Cortés et al. 1993; Sano et al. 1994; Seo et al. 1995).

In summary, the identification of gene expression changes following insect attack is critical to the understanding of plant–insect interactions. This study is also one of only a few plant-defense studies focusing on roots and their responses to insects. We have identified over 150 ESTs from sugar beet roots and have classified them based on their regulation patterns in the F1016 sugar beet genotype that is moderately resistant to SBRM. The data provided here can be compared to data from other root crops, especially tap root crops such as carrot which is also susceptible to Dipteran insect pests (e.g. carrot rust fly), keeping in mind that some of the expression changes identified here may be specific to SBRM feeding. Based on further characterization of the ESTs identified in this study, novel methods of control using conventional breeding and/or transgenic lines will be possible.

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